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# Osteoarthritis and Cartilage

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## Macromer density influences mesenchymal stem cell chondrogenesis and maturation in photocrosslinked hyaluronic acid hydrogels

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### Summary

**Objective:** Engineering cartilage requires that a clinically relevant cell type be situated within a 3D environment that supports cell viability, the production and retention of cartilage-specific extracellular matrix (ECM), and eventually, the establishment of mechanical properties that approach that of the native tissue. In this study, we investigated the ability of bone marrow derived mesenchymal stem cells (MSCs) to undergo chondrogenesis in crosslinked methacrylated hyaluronic acid hydrogels (MeHA) of different macromer concentrations (1, 2, and 5%).

**Design:** Over a 6 week culture period under pro-chondrogenic conditions, we evaluated cartilage-specific gene expression, ECM deposition within constructs and released to the culture media, and mechanical properties in both compression and tension. Further, we examined early matrix assembly and long term histological features of the forming tissues, as well as the ability of macromolecules to diffuse within hydrogels as a function of MeHA macromer concentration.

**Results:** Findings from this study show that variations in macromer density influence MSC chondrogenesis in distinct ways. Increasing HA macromer density promoted chondrogenesis and matrix formation and retention, but yielded functionally inferior constructs due to limited matrix distribution throughout the construct expanse. In 1% MeHA constructs, the equilibrium compressive modulus reached 0.12 MPa and s-GAG content reached nearly 3% of the wet weight, values that matched or exceeded those of control agarose constructs and that are 25 and 50% of native tissue levels, respectively.

**Conclusions:** These data provide new insight into how early matrix deposition regulates long term construct development, and defines new parameters for optimizing the formation of functional MSC-based engineered articular cartilage using HA hydrogels.

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**Key words:** Mesenchymal stem cells, Photocrosslinked hydrogels, Chondrogenesis, Mechanical properties.

### Introduction

Articular cartilage lines the surfaces of joints and functions to transmit stresses. This function is enabled by the complex interplay between the fluid within the tissue and the dense extracellular matrix (ECM); specifically the type II collagen (Col II) network and the negatively charged large proteoglycans (PG). Subsequent to trauma, or as a result of degenerative diseases, cartilage undergoes fluctuations in its mechanical and biochemical content, and thus, loses its load-bearing capacity. To address this, the last two decades have witnessed a surge in activity aimed at the formation of engineered cartilage. Much of this work employed articular chondrocytes in three-dimensional (3D) culture environments (see Ref. 1 for review). In 3D hydrogels, in particular, chondrocytes produce cartilage ECM that is assembled into a functional network with properties that begin to approximate that of native tissue<sup>2</sup>. Physical properties of hydrogels, including polymer density and cross-linking, control the localization and mechanical properties of newly formed matrix<sup>3,4</sup>, as well as the diffusion of large

macromolecules<sup>5</sup>. The potential of hydrogels is underscored by recent work showing that compressive properties can meet or exceed native tissue properties (0.5–1.0 MPa) when custom media regimens are employed<sup>6,7</sup>.

Despite these promising findings, the clinical use of chondrocytes may have limitations. Aged chondrocytes form mechanically inferior constructs when compared to those derived from juvenile chondrocytes<sup>8,9</sup>. An alternative might be the use of mesenchymal stem cells (MSCs)<sup>10–12</sup>, which are expandable and retain their multi-differentiation characteristics<sup>13</sup>. As with chondrocytes, a range of 3D environments has been employed for engineering cartilage with MSCs (e.g.,<sup>14–19</sup>). We recently demonstrated that bovine MSCs undergo chondrogenesis in agarose (a thermoreversible hydrogel), in self-assembling peptide gels, and in photocrosslinked hyaluronic acid (HA) hydrogels<sup>20</sup>. Mechanical properties and biochemical content of these constructs increased with time in each hydrogel, though tissue formation was dependent on the type of hydrogel employed.

The literature on MSC differentiation indicates that specific factors modulate the rate and/or extent of MSC chondrogenesis. The biologic interface can induce different levels of molecular level chondrogenesis and control cell shape; inclusion of Arg-Gly-Asp (RGD) moieties in modified alginate gels can limit chondrogenesis above a certain threshold<sup>21</sup>, while modification of polyethylene glycol (PEG) hydrogels with a collagen mimetic peptide can

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enhance chondrogenesis<sup>22</sup>. Direct comparisons between photocrosslinked PEG hydrogels (simple, non-interactive) and HA hydrogels (biologic, interactions through CD44 receptors) show improved chondrogenesis in HA when all other factors are held constant<sup>23</sup>. Additionally, biophysical properties such as pore size modulate the extent to which MSCs differentiate and accumulate matrix in PEG-based semi-interpenetrating networks<sup>24</sup>. While not yet shown for chondrogenesis in 3D culture, the micromechanics of the supporting environment can also tune MSC lineage-specification in 2D culture systems<sup>25</sup>.

Collectively, these findings suggest that the biological, mechanical, and biophysical properties of the microenvironment interact to control the lineage-specification of MSCs, as well as tissue maturation. Our past work with crosslinked HA hydrogels suggests that macromer density (which is inversely related to pore size and directly proportional to bulk mechanical properties) can be used to tune matrix formation by auricular chondrocytes<sup>26</sup>. As our previous findings show that MSCs undergo chondrogenesis in HA gels, but do so to a lesser extent than in other hydrogel environments (such as agarose), the purpose of this study was to determine whether changes in HA macromer density influence ECM deposition and generation of functional cartilage-like properties by MSCs. Results indicated that increasing HA density promoted chondrogenesis and matrix formation and retention, but yielded functionally inferior constructs due to limited matrix distribution throughout the construct expanse. These data provide new insight into how early matrix deposition regulates long term construct development, and define new parameters for optimizing functional MSC-based engineered cartilage using HA hydrogels.

## Materials and methods

### MSC ISOLATION AND EXPANSION

Bone marrow was extracted from juvenile bovine tibiae (Research 87, Boylston, MA) and MSCs isolated<sup>14</sup>. MSCs were expanded in a basal medium (BM) composed of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS, Gibco Invitrogen, Carlsbad, CA) and 1% penicillin–streptomycin–fungizone (PSF) through 2–3 passages. Three replicate studies were performed, with MSCs from 2 to 3 donor animals pooled for each replicate. Each replicate showed similar trends, and data from one replicate are presented.

### FABRICATION OF ACELLULAR AND MSC-SEEDED CONSTRUCTS

Photocrosslinkable HA macromer was synthesized as previously described<sup>27</sup>. Briefly, 65 kDa HA (Lifecore, Chaska, MN) was methacrylated with methacrylic anhydride (Sigma Chemicals, St. Louis, MO). Degree of methacrylation (assessed by nuclear magnetic resonance<sup>27</sup>) was ~25%. Methacrylated HA (MeHA) solutions were prepared at 1, 2, and 5% (mass/volume) in phosphate-buffered saline (PBS) with 0.05% w/v of the photoinitiator I2959 (2-methyl-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone, Ciba-Geigy, Tarrytown, NY). For cell-laden gels, MSCs were suspended in MeHA solutions at 20 million cells/mL. Acellular and MSC-laden MeHA macromer suspensions were then cast between glass plates separated by a 2.25 mm spacer and photo-polymerized with ultraviolet (UV) exposure<sup>20</sup>. For controls, agarose hydrogels (Ag, 2.25 mm thick) were formed at 20 million cells/mL<sup>28</sup>. Cylindrical constructs were cored from hydrogel slabs at 4 mm (for MSC-laden gels) or 8 mm (for acellular gels).

### MECHANICAL CHARACTERIZATION OF ACELLULAR CONSTRUCTS

Acellular MeHA disks (5 mm diameter by 2.25 mm thickness) were formed as above and tested in confined compression in a PBS bath<sup>29</sup>. Three sequential ramps of 10% strain (0.05%/s) were applied, and samples were allowed to reach equilibrium between ramps (~1200 s). Data from the second ramp (10–20% deformation) were extracted and fit to the Biphasic Theory of

Mow and co-workers<sup>30</sup> to determine construct permeability ( $k$ ) and aggregate modulus ( $H_A$ ).

### MACROMOLECULAR DIFFUSION IN ACELLULAR CONSTRUCTS

Fluorescein-conjugated dextran (70 and 2000 kDa; Molecular Probes, Invitrogen) was suspended within MeHA (1, 2, and 5%) hydrogels at 175  $\mu$ g/mL or 85  $\mu$ g/mL, respectively. Gels were maintained in 2 mL of PBS at 37°C on a rocker plate, and supernatant sampled over 72 h. Released dextran was measured *via* fluorescence (485 nm/518 nm), with concentration determined from standard curves. The effective 'diffusivity' was determined by plotting concentration (normalized to final) vs the square root of time<sup>31</sup>.

### LONG TERM CULTURE CONDITIONS FOR MSC-SEEDED CONSTRUCTS

Constructs were cultured for up to 6 weeks (1 mL/construct) in transforming growth factor beta 3 (TGF- $\beta$ 3) (10 ng/mL; R&D Systems, Minneapolis, MN) supplemented, chemically defined medium (CM+). CM consisted of high-glucose DMEM with 1  $\times$  PSF, 0.1  $\mu$ M dexamethasone, 50  $\mu$ g/mL ascorbate 2-phosphate, 40  $\mu$ g/mL L-proline, 100  $\mu$ g/mL sodium pyruvate, and insulin–transferrin–selenium + (ITS+), 6.25 mg/mL insulin, 6.25 mg/mL transferrin, 6.25 ng/mL selenous acid, 1.25 mg/mL bovine serum albumin (BSA), and 5.35 mg/mL linoleic acid). Media were changed twice weekly.

### VIABILITY AND SHORT TERM EXPRESSION ANALYSIS OF MSC-SEEDED CONSTRUCTS

For viability assays, samples were tested at 3 and 6 weeks using the LIVE/DEAD staining kit (Molecular Probes, Invitrogen)<sup>20</sup>. Additionally, on days 0, 3, and 21, metabolic activity was quantified with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay<sup>20</sup>. Briefly, samples were incubated in MTT reagent for 1 h at 37°C, washed in PBS, developed color eluted with dimethyl sulfoxide (DMSO), and absorbance read at 540 nm. For gene expression, RNA was extracted from day 0, 1, 7 and 21 samples with two sequential extractions in TRIzol–chloroform. After quantification of RNA yield and purity (Nanodrop, Thermo Scientific, Waltham, MA), reverse transcription was carried out with the Superscript First Strand Synthesis System kit (Invitrogen). cDNA amplification was carried out using SYBR Green Master Mix on a 7300 Applied Biosystems real time polymerase chain reaction (PCR) machine with intron spanning primers. Expression of type I collagen (Col I), Col II and aggrecan (AGG) was determined and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

### BIOMECHANICAL ANALYSIS OF MSC-SEEDED CONSTRUCTS

Compressive equilibrium ( $E_v$ ) and dynamic (IG\* $I$ ) moduli of constructs were determined by unconfined compression between impermeable platens in a PBS bath<sup>29</sup>. Compressive modulus was determined *via* stress-relaxation testing, and subsequently a 1.0 Hz sinusoidal deformation of 1% was applied and dynamic modulus determined<sup>32</sup>. In a separate study, tensile properties were measured. MSC-seeded samples were fabricated as above, but cut from slabs into strips (4 mm  $\times$  20 mm  $\times$  1.5 mm), and cultured in CM+ (6 mL per strip). Given the larger size of these samples and the poor findings with 5% HA in compression studies, only 1 and 2% MeHA concentrations were investigated. Samples were tested *via* a quasi-static extension to failure<sup>28</sup>, with the ramp tensile modulus computed from the linear region of the stress–strain curve.

### BIOCHEMICAL ANALYSIS OF MSC-SEEDED CONSTRUCTS

After testing, construct wet weights were recorded, and DNA, sulfated glycosaminoglycan (s-GAG), and collagen contents assessed<sup>20</sup>. DNA and s-GAG content were determined *via* Picogreen (Molecular Probes, Eugene, OR) and 1,9-dimethylmethylene blue (DMMB) assays, respectively<sup>33</sup>. Orthohydroxyproline (OHP) content of hydrolyzed digest was measured *via* reaction with chloramine T and diaminobenzaldehyde<sup>34</sup>. Collagen was extrapolated from OHP using a 1:7.14 ratio of OHP:collagen<sup>35</sup>. In one replicate, s-GAG content of culture medium was measured at each feeding.

### HISTOLOGICAL ANALYSIS OF MSC-SEEDED CONSTRUCTS

Constructs were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned (8  $\mu$ m). Analysis was carried out on days 3, 5, 7, 10, and 14 and bi-weekly through week 6. Samples were stained for PG with Alcian blue (pH 1.0) and for collagen *via* Picrosirius red<sup>28</sup>. Immunohistochemistry was used to visualize localization of Col I and Col II<sup>28</sup>. Samples underwent

antigen retrieval and were sequentially treated at room temperature with 300 mg/mL hyaluronidase (Type IV, Sigma, St. Louis, MO), 3% H<sub>2</sub>O<sub>2</sub>, and blocking reagent (DAB150 IHC Select, Millipore, Billerica, MA). Sections were then treated with antibodies (5 µg/mL) to Col I (MAB3391, Millipore) or Col II (11e-116B3, Developmental Studies Hybridoma Bank, Iowa City, IA) in 3% BSA (control sections treated with 3% BSA only). Finally, biotinylated goat anti-rabbit IgG secondary antibody conjugated with streptavidin horseradish peroxidase was localized to primary antibodies, and color developed with 3,3'-diaminobenzidine (DAB) chromagen reagent (DAB150 IHC Select, Millipore). Images were acquired at magnifications of 5 or 10×.

#### STATISTICAL ANALYSIS

Data are reported as the mean and standard deviation (SD); sample numbers are indicated in the associated figure legends. Statistical analysis (SYSTAT 12, Systat Software, Chicago, IL) included both one-way and two-way ANOVA, with gel group (1%, 2%, 5%, Ag) and time in culture as independent variables. When significance ( $P < 0.05$ ) was indicated by ANOVA, Tukey's *post hoc* tests were applied to enable comparisons between groups.

## Results

#### MACROMER DENSITY INFLUENCES ACELLULAR HYDROGEL MECHANICS

Prior to cell-seeding studies, crosslinked MeHA hydrogels were formed at varying concentrations (1, 2, and 5%) and tested in confined compression. Increasing macromer concentration led to decreases in construct permeability ( $k$ ), with both 2 and 5% MeHA hydrogels significantly less permeable than 1% MeHA hydrogels ( $P < 0.05$ ; Fig. 1). HA showed the reverse trend, with 5% MeHA gels significantly stiffer than both 1 and 2% gels ( $P < 0.05$ ).

#### MSC VIABILITY AND DIFFERENTIATION IN HA GELS WITH INCREASING MACROMER DENSITY

After ascertaining concentration-dependent differences in hydrogel properties, MSC viability and differentiation was assessed in MeHA gels of increasing macromer concentration (1, 2, and 5%). Viable cells were observed uniformly in all MeHA and Ag constructs on both day 21 (Fig. 2) and day 42 (not shown). There appeared to be more cell clustering with higher MeHA concentrations at both time points. Little evidence of cell death was observed under any condition. Metabolic activity showed that, relative to day 1, 1% MeHA and Ag gels increased with time ( $P < 0.05$ ), but after 21 days no significant differences were observed between groups ( $P > 0.05$ ). DNA content per construct was ~20 and ~40% higher after 42 days in 2 and 5% MeHA hydrogels compared to 1% MeHA and Ag hydrogels, respectively ( $P < 0.05$ ).

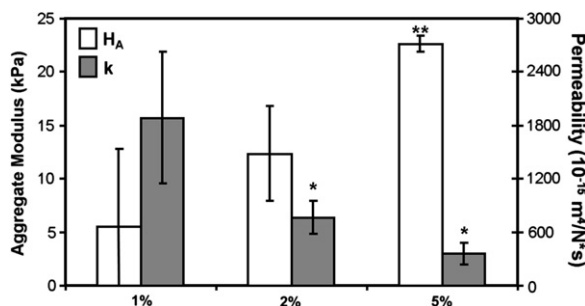


Fig. 1. Biphasic parameters of permeability ( $k$ ) and aggregate modulus ( $H_A$ ) for MeHA gels with increasing macromer density. ( $R^2 > 0.89$ ;  $n = 3-4$ /group; \* indicates  $P < 0.05$  vs 1%; \*\* indicates  $P < 0.05$  vs 1 and 2%)

Expression analysis was performed on MSC-seeded constructs maintained in pro-chondrogenic medium (CM+). Results indicated that Col I expression remained low throughout the 21 day period, at most increasing by a factor of two over this time course. Conversely, Col II expression increased dramatically in each condition, and appeared to be a function of macromer density (with levels in 5% MeHA nearly fourfold greater than in 1% MeHA or Ag; Fig. 3). AGG increased relative to starting levels in each construct by day 7, with generally higher levels of expression observed in the MeHA constructs compared to Ag constructs. For AGG, no clear differences were observed between MeHA gels of different concentrations. These data indicate that MSCs are viable in MeHA hydrogels over long periods, that constructs have stable or slightly increased cell content, and that MSCs undergo chondrogenesis in each of these 3D environments.

#### CONSTRUCT DIMENSIONAL STABILITY AND BIOCHEMICAL CONTENT

Biochemical content in engineered cartilage is a function of matrix deposition and retention, as well as volumetric space. In low concentration MeHA gels, initial dimensions (diameter and thickness) decreased markedly (Fig. 4). This contraction occurred in both acellular and MSC-seeded gels, suggesting that the initial contraction is a function of the gel itself, rather than cell-mediated mechanisms. Acellular and MSC-seeded 1% MeHA constructs contracted by ~10% in thickness and ~20% in diameter over the first day. Conversely, 5% gels increased in thickness by ~5%, with no change in diameter. 2% MeHA constructs were intermediate to these extremes, while Ag constructs did not change, consistent with previous findings. With culture, construct dimensions changed as well; 1% MeHA constructs recovered towards their original geometry, while 2 and 5% MeHA constructs increased in thickness by ~30%, and in diameter by 10–20%, by day 42. Over this same time course, Ag constructs showed small increases in diameter and thickness.

Biochemical content of constructs was assessed through 6 weeks of culture. On a per wet weight (ww) basis, 1% MeHA constructs accumulated the highest s-GAG content, reaching levels comparable to Ag constructs ( $P > 0.05$ ), while 2 and 5% MeHA constructs contained less s-GAG [ $P < 0.05$ ; Fig. 5(A)]. On day 42, 1% MeHA and Ag constructs contained ~3% s-GAG per wet weight, while 2 and 5% MeHA constructs contained ~2% s-GAG. Conversely, in terms of total s-GAG per construct, values in 1% MeHA constructs were less than both 2 and 5% MeHA constructs ( $P < 0.05$ ; Table I). s-GAG lost to the culture media was highest for Ag, reaching peak release rates by day 11. 5% MeHA constructs released the least amount s-GAG per day over the first 21 days, with similar release rates from each MeHA construct observed thereafter [Fig. 5(C)]. Collagen content showed a similar trend as s-GAG, with the exception of Ag constructs, which contained higher collagen levels (1.4% ww,  $P < 0.05$ ) than each of the MeHA constructs by day 42 [1%: 0.7%ww, 2%: 0.3%ww, 5%: 0.4%ww; Fig. 5(B)]. In terms of total collagen per construct, Ag constructs contained the highest levels, while the MeHA formulations were not different from one another (Table I).

#### MECHANICAL PROPERTIES OF MSC-LADEN CONSTRUCTS

Differences in biochemical content (and concentration) of ECM resulted in widely different compressive and tensile



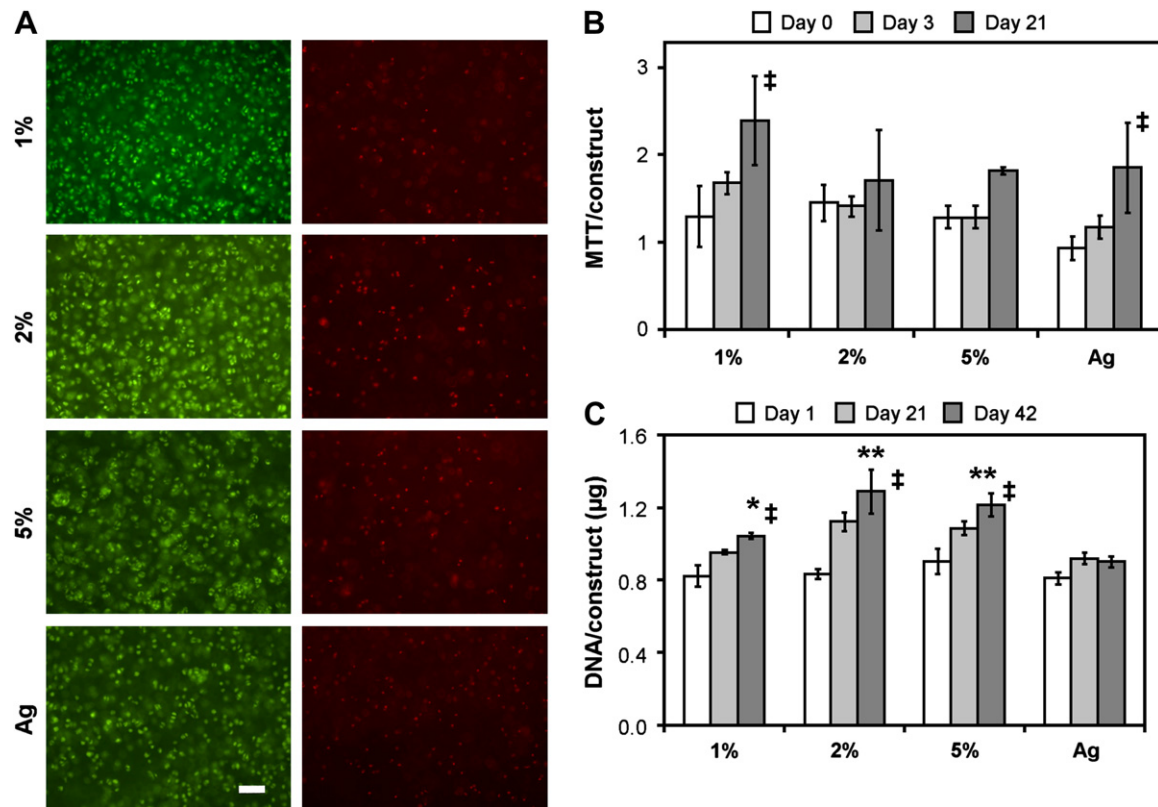


Fig. 2. (A) Live (green, left) and dead (red, right) MSCs in 1, 2, and 5% MeHA, and Ag hydrogels 21 days after encapsulation (10 $\times$  magnification; 200  $\mu$ m scale bar). (B) Mitochondrial activity of constructs through day 21. (C) DNA content of MSC-seeded constructs through day 42. ( $n = 4$ /group/time point, \*\* indicates  $P < 0.05$  vs 1% and Ag on day 42, \* indicates  $P < 0.05$  vs Ag on day 42; ‡ indicates  $P < 0.05$  vs day 0)

properties. While all constructs increased in equilibrium and dynamic modulus with time ( $P < 0.05$ ), by day 42 the equilibrium modulus of 1% MeHA constructs was  $\sim 20\%$  greater than Ag constructs [ $P < 0.05$ ; Fig. 6(A)] and more than 100% greater than both 2 and 5% MeHA constructs ( $P < 0.05$ ). The dynamic compressive modulus data followed a similar trend, where 1% MeHA constructs were  $\sim 20\%$  greater than Ag, and  $\sim$  fivefold greater than 2 and 5% MeHA constructs [ $P < 0.05$ ; Fig. 6(B)]. On day 42, the tensile modulus of Ag constructs was  $\sim$  twofold higher than that of 1% MeHA [ $P < 0.05$ ; Fig. 6(D)], while 1% MeHA constructs were more than sevenfold greater than 2% MeHA constructs ( $P < 0.05$ ). Failure strain did not change markedly with culture [Fig. 6(C)].

#### ECM DEPOSITION AND DISTRIBUTION IN MSC-LADEN CONSTRUCTS

Histological analysis at early time points showed marked differences in matrix distribution as a function of macromer density (Fig. 7). In 5% MeHA constructs, PGs were sequestered into dense rings around cells by day 7. In contrast, 1% MeHA and Ag gels showed a more homogenous distribution of PG. Similarly, by day 42, PG and collagen were evenly distributed in 1% MeHA and Ag, while intense pericellular localization was evident in 5% MeHA constructs (Fig. 8). Col II staining (Fig. 8) showed increased sequestration of this ECM component in the pericellular space in higher macromer concentration MeHA constructs. Consistent with biochemical findings, histological results also

show more intense collagen (bulk and type II) staining in Ag constructs compared to all MeHA constructs.

#### MACROMOLECULAR DIFFUSION IN ACELLULAR MeHA HYDROGELS

To better understand the mechanism of matrix distribution, we evaluated macromolecular diffusivity of small (70 kDa, on the order of growth factors) and large (2000 kDa, on the order of ECM aggregates) molecules in MeHA gels of varying macromer density. Release rates of 70 kDa dextran from MeHA hydrogels decreased as macromer density increased [Fig. 9(A)]. A similar finding was observed with 2000 kDa dextran [Fig. 9(B)]. Linear regression to the relative concentration plotted against the square root of time provides a quantitative 'effective diffusivity' for comparing these responses. Linear fits captured the data well ( $R^2 > 0.75$ ) for each macromer density and both dextran sizes. Macromer concentration had a significant effect on 'effective diffusivity' for both 70 and 2000 kDa dextran. For each increase in macromer concentration, a significant decrease in diffusivity was observed [ $P < 0.05$ ; Fig. 9(C)].

#### Discussion

Realization of a functional engineered cartilage construct requires that a clinically relevant cell type be situated within a 3D environment that supports cell viability as well as the production and retention of cartilage-specific ECM molecules. Further, the encapsulating material must allow

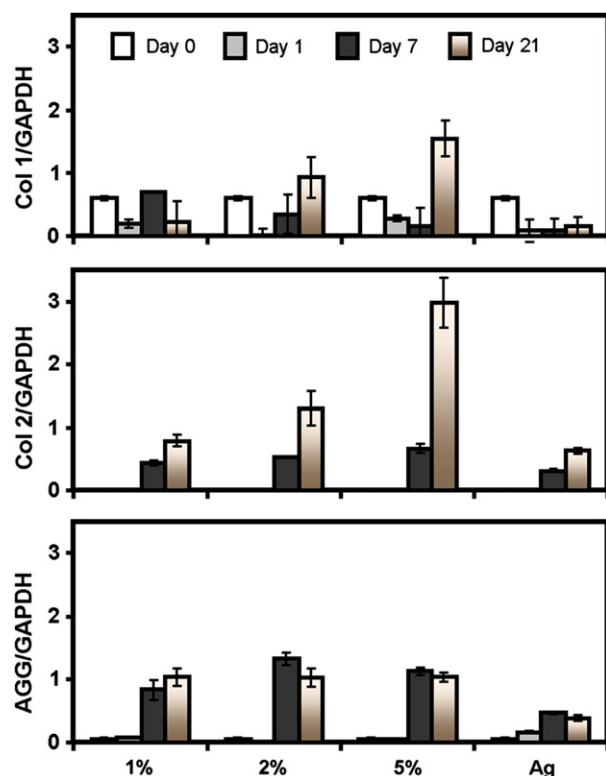


Fig. 3. Col I (top), Col II (middle), and AGG (bottom) mRNA levels in MSC-seeded MeHA (1, 2 and 5%) and Ag constructs through 21 days of chondrogenic culture. Note modest changes in Col I expression with time and robust increases in Col II and AGG, indicative of chondrogenic differentiation.

assembly of these molecules into a dense network with physiologic mechanical properties. In this work, we investigated the ability of MSCs to undergo chondrogenesis in crosslinked MeHA hydrogels. This hydrogel formulation has several promising attributes; it is a well defined biologic that can be photo-polymerized *in situ* to fill any sized defect<sup>36</sup>. Our previous studies with this gel at one macromer concentration (2%) established that MSCs undergo chondrogenesis in MeHA, but also indicated that the rate and extent of functional maturation was reduced when compared to agarose<sup>20</sup>. As MeHA macromer density influences ECM deposition by auricular chondrocyte-seeded HA gels<sup>26</sup>, this study specifically investigated how variations in this parameter influence the maturation of MSC-based

constructs. Results from this study demonstrated that two competing effects occur as macromer density increases: enhanced chondrogenesis that is countermanded by biophysical impediments to distributed matrix assembly.

As previously noted for MeHA<sup>26</sup>, and consistent with other hydrogels<sup>37</sup>, changes in macromer density had marked effects on the mechanical properties; constructs with higher macromer densities were stiffer. Despite the increasing gel density, viability and DNA assays indicated that cells survive and divide throughout the material. Encapsulated MSCs increased expression of cartilage-specific matrix and accumulated increasing amounts of PG through 42 days. Despite the hindered diffusion observed for 70 kDa molecules, histological analysis (staining for PG deposition) showed that chondrogenesis occurred throughout the gel, and was not restricted to the periphery in higher macromer concentrations. These findings suggest that the HA gels support viability and MSC chondrogenesis at all macromer concentrations.

The enhanced chondrogenic differentiation and PG observed in higher macromer concentration MeHA constructs could arise from a number of different factors. First, we have previously shown that MeHA hydrogels enhance molecular level chondrogenesis compared to inert crosslinked networks such as PEG<sup>23</sup>. HA is a component of the native cartilage ECM and so the gel presents a biologic interface with which both chondrocytes and MSCs can interact (through CD44 receptors)<sup>38</sup>. In high macromer constructs, a greater probability of receptor mediated interaction with the material exists, just as concentration-dependent effects are observed when RGD is coupled to otherwise biologically inert hydrogels<sup>21</sup>. Alternatively, the higher stiffness of the material may influence differentiation. Findings in monolayer studies suggest that MSCs can interpret the microenvironmental stiffness to modulate differentiation<sup>39</sup>. Here, increasing macromer density increases gel stiffness; MSCs may respond to this by increasing the degree to which they undergo chondrogenesis. Still another possibility relates to the rapid and intense accumulation of newly formed matrix in the pericellular space in higher density MeHA. While a high local PG concentration exerts negative feedback on further PG production by chondrocytes<sup>2</sup>, this does not seem to be the case with MSCs in this system. Rather, the ECM in the pericellular space may act to concentrate locally produced factors (ECM to which the cells bind, or growth factors that themselves bind to ECM), creating a microenvironment that better supports and/or maintains chondrogenesis. Future studies will be required to elucidate the precise mechanism by which this enhanced differentiation occurs in higher density MeHA gels.

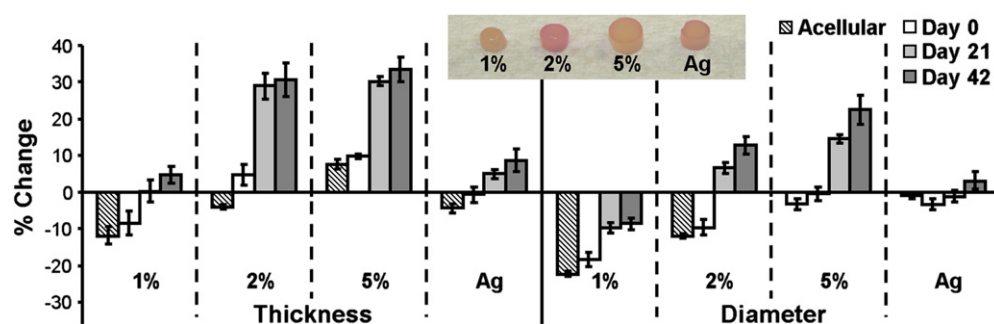


Fig. 4. Dimensional variation in acellular and MSC-seeded constructs with time in culture. Differences shown as the percentage of initial size (4 mm diameter and 2.25 mm thickness,  $n = 4$ /group/time point). Inset image of MSC-seeded constructs after 6 weeks of *in vitro* culture in chondrogenic medium.

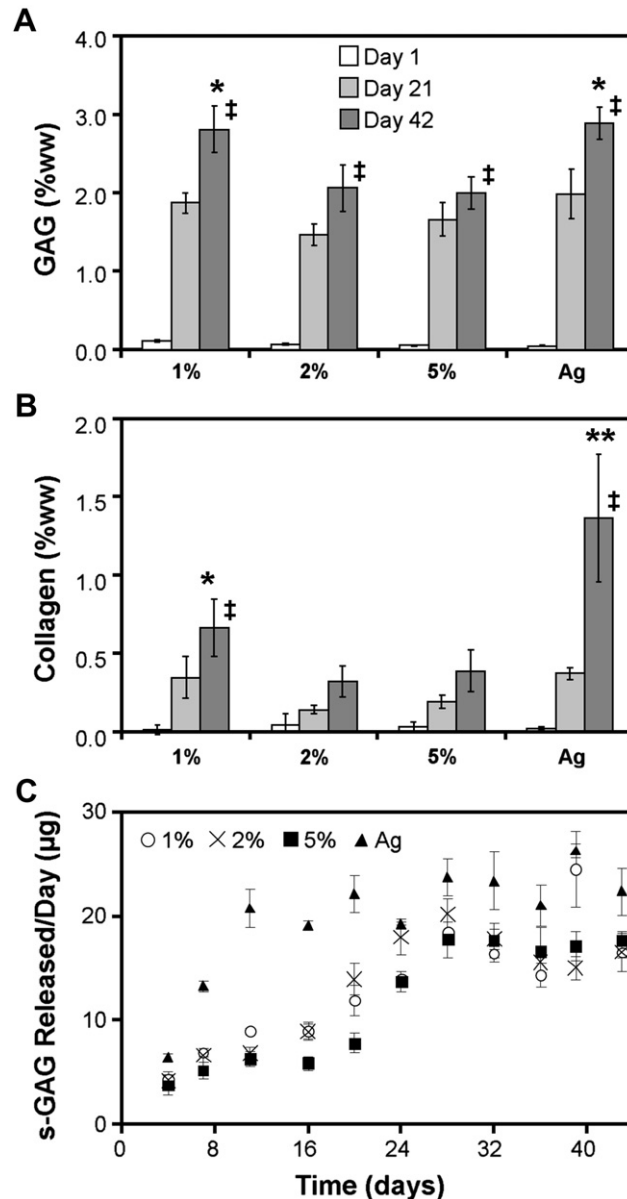


Fig. 5. (A) s-GAG percent wet weight (% ww) in 1, 2, and 5% MeHA, and Ag constructs through 42 days of *in vitro* chondrogenic culture. (\* indicates  $P < 0.05$  vs 2 and 5% MeHA at day 42) (B) Collagen content (% ww) in MeHA and Ag constructs through 42 days of culture. (\*\* indicates  $P < 0.05$  vs all other groups at day 42, \* indicates  $P < 0.05$  vs 2% MeHA) Increased concentration of ECM was observed in Ag and 1% MeHA hydrogels by day 42. ( $n = 4$ /group/time point, ‡ $P < 0.05$  vs day 0) (C) s-GAG release per day per construct for MSC-seeded MeHA and Ag constructs through 42 days of culture.

Despite the anabolic and/or pro-chondrogenic effects of increasing MeHA macromer density, these positive findings were counterbalanced by the limited diffusion of large ECM molecules away from their origin. This limitation impeded the homogenous distribution of formed ECM, and so hampered the functional maturation. This is consistent with the findings of Buxton *et al.*, who showed that inclusion of spacers within PEG gels allowed for greater matrix distribution by human MSCs<sup>24</sup> and by Ng *et al.* using bovine chondrocytes in an agarose system<sup>37</sup>. While 5% MeHA constructs produced and retained the highest absolute amount of PG, they failed to develop increasing mechanical properties compared to lower macromer concentration constructs that produced lesser amounts of PG. This is partially

due to volumetric changes observed; 1% MeHA constructs made less PG, but contracted slightly and so concentrated the formed ECM, while 5% MeHA constructs made and retained more PG, but swelled significantly. These findings suggest that new methods must be developed to take advantage of the positive features of a higher MeHA concentration, while increasing the mobility of newly formed matrix. For example, Bryant *et al.* have shown greater ECM distribution in chondrocyte-seeded PEG gels that contain degradable linkages<sup>40</sup>, and Park *et al.* have shown similar findings in matrix metalloproteinase (MMP)-cleavable hydrogels<sup>41</sup>. Working with a new hydrolytically degradable version of these crosslinked HA hydrogels, we have recently shown that crosslink degradation leads to more rapid

Table I  
Dimensional, biochemical, and mechanical properties of MSC-seeded MeHA and Ag constructs after 6 weeks of culture (mean  $\pm$  SD; n = 3–4/group)

		1% MeHA		2% MeHA		5% MeHA		2% Agarose	
		Mean $\pm$ SD		Mean $\pm$ SD		Mean $\pm$ SD		Mean $\pm$ SD	
Dimensions	Thickness (mm)	2.36	0.05	2.94	0.10	3.01	0.08	2.45	0.07
	Diameter (mm)	3.66	0.06	4.52	0.10	4.90	0.16	4.12	0.10
	Wet weight (mg)	26.35	0.72	49.92	1.83	62.04	0.75	33.07	1.05
Biochemistry	DNA per construct ( $\mu$ g)	1.04	0.02	1.29	0.12	1.22	0.06	0.90	0.03
	Collagen per DNA	167.40	45.50	124.78	40.56	203.46	75.80	499.68	142.89
	Collagen per construct ( $\mu$ g)	175.17	49.49	160.66	52.55	240.18	80.85	444.79	132.61
	Collagen (% ww)	0.66	0.18	0.32	0.10	0.39	0.13	1.37	0.41
	GAG per DNA	707.73	54.25	800.47	99.27	1019.45	70.36	1058.92	73.77
	GAG per construct ( $\mu$ g)	739.14	62.73	1024.67	107.62	1241.54	132.43	956.25	88.50
	GAG (% ww)	2.81	0.30	2.06	0.30	2.00	0.20	2.89	0.21
Mechanics	$E_v$ (kPa)	136.66	17.07	53.57	9.78	60.80	8.25	111.96	23.96
	IG*1 (MPa)	1.05	0.12	0.21	0.05	0.16	0.03	0.83	0.13
	Tensile Modulus (kPa)	473.60	187.52	63.50	36.78	—	—	991.48	162.25

dispersion of ECM in short term MSC studies<sup>42</sup>. It is not yet clear how long the pro-chondrogenic signal provided by the HA microenvironment (be it stiffness or biologic moieties) must be present to result in long term increases in matrix production. In future studies, it will be critical to carefully tune early matrix assembly, and the positive benefits thereof, with long term requirements for matrix elaboration.

The results of this study are promising, in that they show a clear macromer density dependent development of construct mechanical properties. The equilibrium and dynamic

compressive properties of MSC-seeded 1% MeHA constructs match or exceed properties achieved with Ag hydrogels seeded with the same MSC population and maintained identically. Indeed, equilibrium compressive properties and s-GAG content reach 25 and 50% of native tissue levels, respectively. However, collagen content in MeHA gels remains low, and is lower than that produced in Ag constructs. This is a significant finding, as collagen content correlates well with tensile properties in native tissue and engineered constructs. In this study, the tensile properties

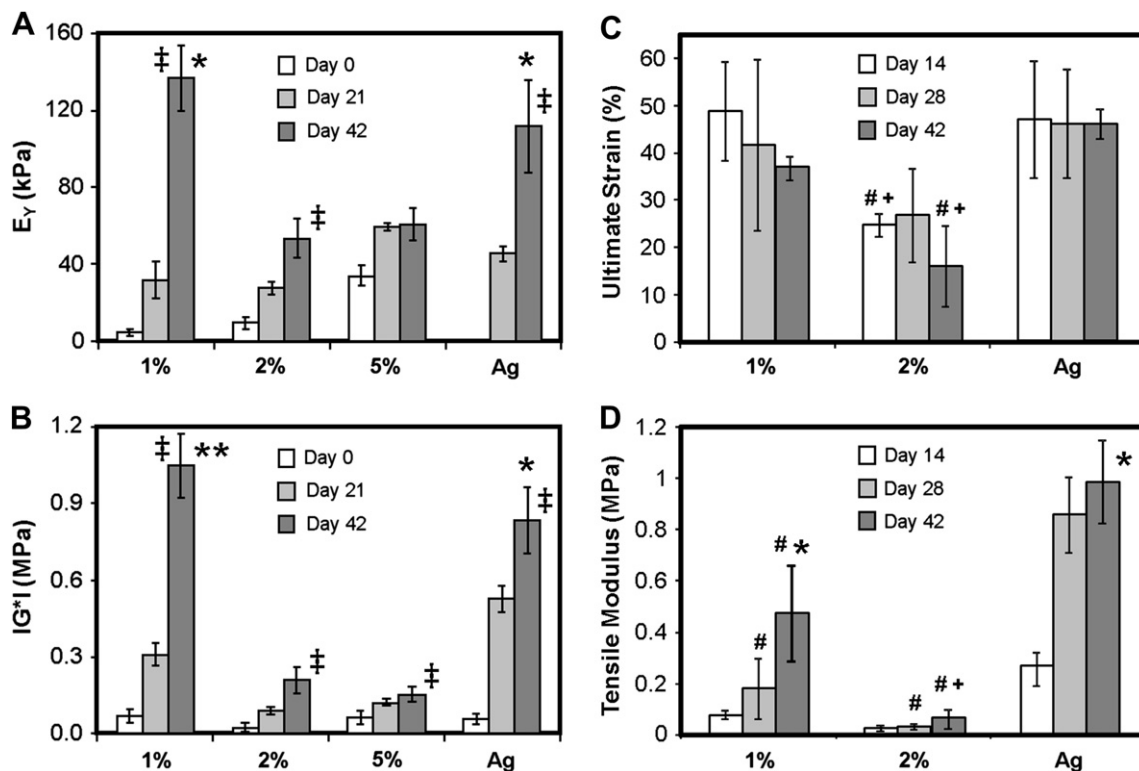


Fig. 6. Equilibrium compressive modulus (A) and dynamic modulus (B) of MeHA and Ag hydrogels through 6 weeks of culture (\*\* indicates  $P < 0.05$  vs all other groups at day 42; \* indicates  $P < 0.05$  vs 2 and 5% MeHA). Failure strain (C) and tensile modulus (D) of MSC-seeded 1 and 2% MeHA and Ag constructs at 2, 4, and 6 weeks. (\* indicates  $P < 0.05$  vs all other groups on the terminal time point (day 42); # indicates  $P < 0.05$  vs Ag group at same time point; + indicates  $P < 0.05$  vs 1% MeHA group at same time point) Biomechanical properties increase more rapidly and to a higher level in lower concentration MeHA constructs. (n = 4/group/time point, ‡  $P < 0.05$  vs day 0)



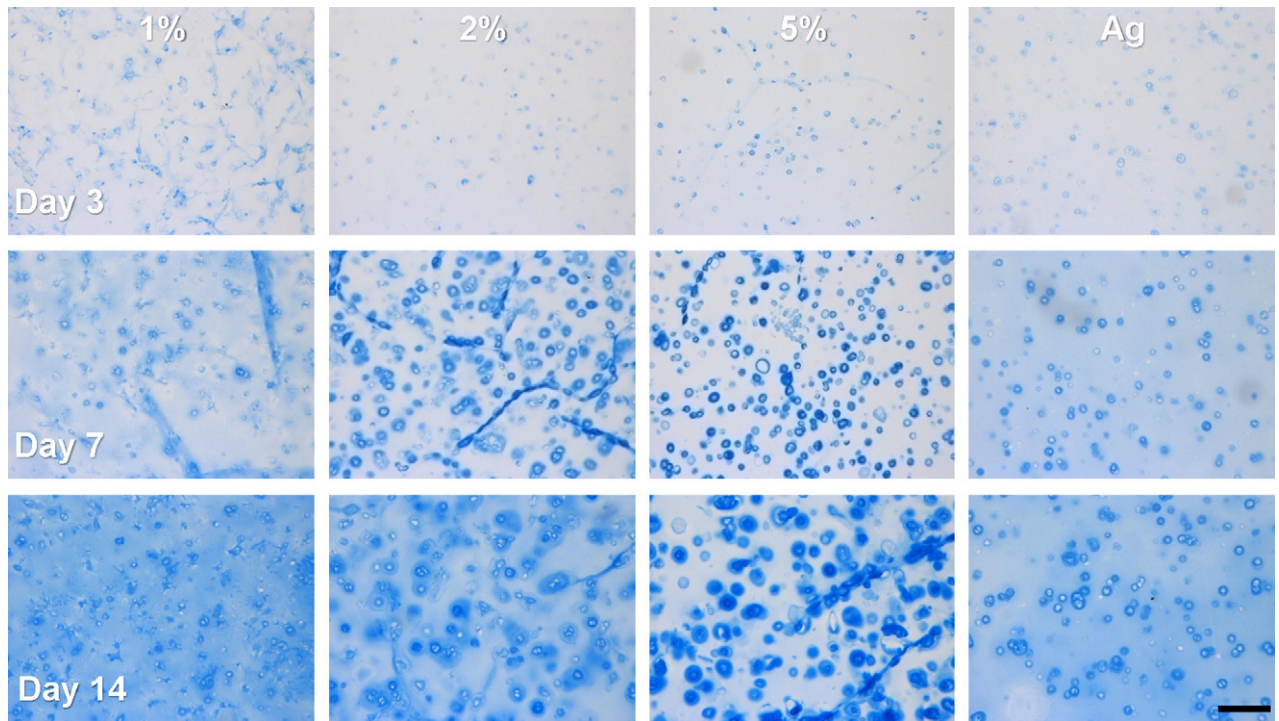


Fig. 7. Alcian blue stained sections of MSC-seeded 1, 2, and 5% MeHA and Ag constructs after 3 (top), 7 (middle), and 14 days (bottom) of chondrogenic culture (10 $\times$  magnification). Pericellular aggregation of PG is evident in higher % MeHA constructs in contrast to a more even distribution in 1% MeHA constructs and Ag controls. (Scale bar = 250  $\mu$ m)

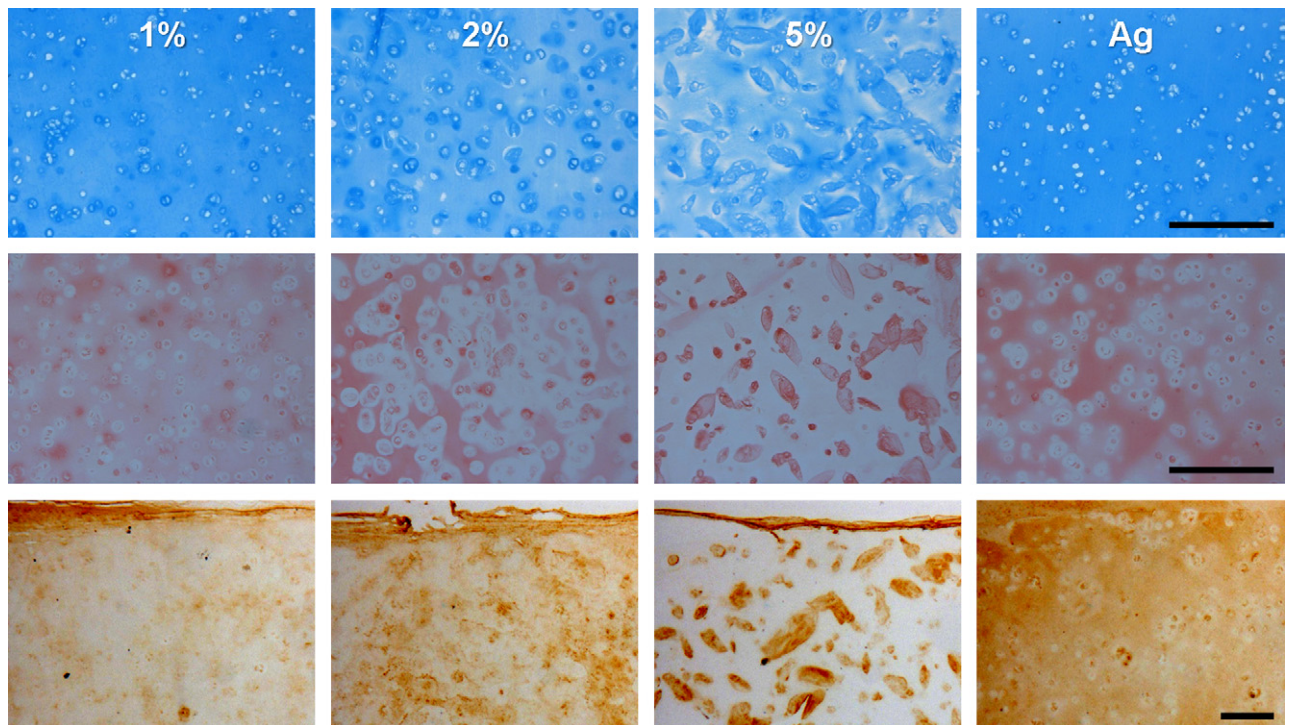


Fig. 8. Alcian blue (top) and Picrosirius red (middle) stained sections from 1, 2, and 5% MeHA and Ag constructs (10 $\times$  magnification) on day 42. Col II immunostaining (bottom) on day 42 (5 $\times$ ). Note the dependence of PG and collagen distribution on MeHA macromer concentration. (Scale bar = 250  $\mu$ m)



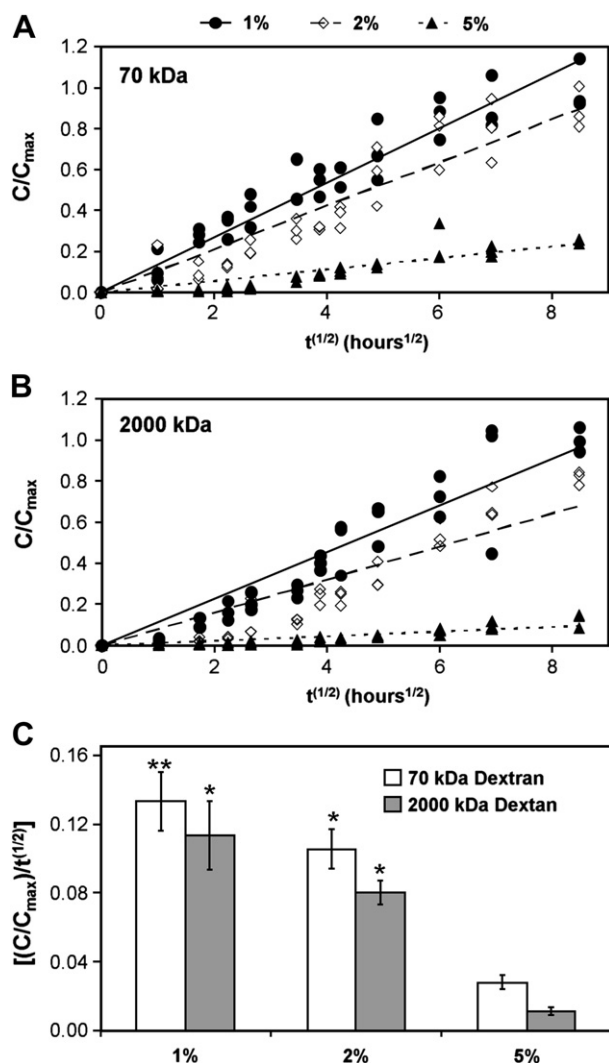


Fig. 9. Time course of release of 70 kDa (A) and 2000 kDa (B) fluorescein-conjugated dextran from 1, 2, and 5% MeHA hydrogels (normalized to maximum concentration from 1% MeHA for a given dextran molecular weight). Effective diffusivity (C) of dextran of both sizes decreased with increasing MeHA macromer concentration. ( $n = 3$ /group; \*\* indicates  $P < 0.05$  vs both 2 and 5% MeHA groups; \* indicates  $P < 0.05$  vs the 5% MeHA group only)

of 1% MeHA constructs remained significantly lower than Ag constructs. Further, it should be noted that the compressive mechanical properties (even in Ag hydrogels) remain lower than that produced by native chondrocytes in Ag hydrogels<sup>14</sup>. This is consistent with the idea that MSCs remain incompletely (or inefficiently) committed to the chondrocyte phenotype, even in MeHA.

Despite these limitations, these data provide new insight into how early matrix deposition regulates long term construct development, and define new parameters for optimizing the formation of functional MSC-based engineered cartilage using HA hydrogels. For example, in the case of higher density MeHA constructs, dynamic loading might be used to further matrix distribution. Theoretical and experimental results suggest that dynamic loading can expedite the movement of large molecules in dense hydrogels<sup>5,43</sup>. Such an approach may be useful in coupling the pro-chondrogenic/matrix formation events in

MeHA hydrogels, while still providing a mechanism for distribution of newly formed constituents throughout the construct, potentially improving bulk mechanical properties. Taken together, these results provide new evidence that HA hydrogels support the functional chondrogenesis of MSCs, with mechanical properties matching or exceeding our best results to date in other hydrogel systems. With further optimization, this material holds tremendous promise in the fabrication of functional cartilage replacements to restore function to damaged or diseased native tissue.

### Conflict of interest

The authors have no conflict of interest.

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